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Kinetochore Recruitment of Two Nucleolar Proteins Is Required for Homolog Segregation in Meiosis I

Kirsten P. Rabitsch,[1,4] Mark Petronczki,[1,4] Jean-Paul Javerzat,[2] Sylvie Genier,[2] Barbara Chwalla,[1] Alex Schleiffer,[1] Tomoyuki U. Tanaka,[3] and Kim Nasmyth[1,*]

[1]Research Institute of Molecular Pathology
Dr. Bohr-Gasse 7
A-1030 Vienna
Austria

[2]Institut de Biochimie et Génétique Cellulaires
Centre National de la Recherche Scientifique
Unité Mixte de Recherche 5095
1 Rue Camille Saint Saëns
33077 Bordeaux
France

[3]School of Life Sciences
University of Dundee
MSI/WTB Complex
DD1 5EH Dundee
United Kingdom

Summary

Halving of the chromosome number during meiosis I depends on the segregation of maternal and paternal centromeres. This process relies on the attachment of sister centromeres to microtubules emanating from the same spindle pole. We describe here the identification of a protein complex, Csm1/Lrs4, that is essential for monoorientation of sister kinetochores in *Saccharomyces cerevisiae*. Both proteins are present in vegetative cells, where they reside in the nucleolus. Only shortly before meiosis I do they leave the nucleolus and form a "monopolin" complex with the meiosis-specific Mam1 protein, which binds to kinetochores. Surprisingly, Csm1's homolog in *Schizosaccharomyces pombe*, Pcs1, is essential for accurate chromosome segregation during mitosis and meiosis II. Csm1 and Pcs1 might clamp together microtubule binding sites on the same (Pcs1) or sister (Csm1) kinetochores.

Introduction

Genome propagation depends not only on DNA replication but also on the segregation of sister DNA molecules to opposite poles of the cell prior to its cytokinesis. In eukaryotic cells, this process, known as mitosis, is mediated by DNA's attachment to microtubules via large proteinaceous structures called kinetochores. Sister DNA molecules (chromatids) must be pulled in opposite directions due to the prior attachment of sister kinetochores to microtubules oriented toward opposite spindle poles. This is known as biorientation or amphitelic attachment. In most organisms, kinetochores contain multiple microtubule binding sites, and cells must therefore ensure that all sites on a given chromatid attach to microtubules with the same orientation. Attachment of different sites on the same chromatid to opposite poles is called merotelic attachment and must be avoided. Monotelic attachment (attachment of only one chromatid to the microtubules) and syntelic attachment (attachment of both sisters to microtubules with the same orientation) must also be avoided (Rieder and Salmon, 1998).

Efficient amphitelic attachment depends on the interconnection of sister chromatids by a multisubunit cohesin complex (Tanaka et al., 2000) and the action of a protein kinase called Ipl1/Aurora B (Tanaka et al., 2002). By resisting the forces exerted by microtubules, cohesin ensures that traction produces stretching of chromatin (He et al., 2000) in the vicinity of kinetochores only if they have bioriented. It has been suggested that Ipl1/Aurora B acts by eliminating kinetochore-microtubule attachments that do not produce such stretching (tension; Tanaka et al., 2002).

Only when all pairs of sister chromatids have been brought under tension in the middle of the cell (metaphase) is their segregation to opposite poles (anaphase) triggered by the destruction of cohesion along their entire length due to cleavage of cohesin's Scc1 subunit by a cysteine protease called separase (Uhlmann et al., 2000; Waizenegger et al., 2000). Scc1 cleavage is regulated by at least three mechanisms: by phosphorylation of its substrate Scc1 by Polo-like kinases, which enhances cleavage (Alexandru et al., 2001); by phosphorylation of separase itself by cyclin B/Cdk1 kinases, which inhibits separase activity (Stemmann et al., 2001); and by the binding of separase to an inhibitory chaperone called securin (Pds1 in yeast; Ciosk et al., 1998; Funabiki et al., 1996). Separase is activated by the sudden proteolysis of both cyclin B and securin by a ubiquitin protein ligase called the anaphase-promoting complex or cyclosome (APC/C; Zachariae and Nasmyth, 1999). A surveillance mechanism called the spindle checkpoint prevents this from occurring while there still exist kinetochores unoccupied by microtubules or chromatid pairs that have failed to come under tension (Nasmyth, 2002).

Asexual reproduction relies on alternating rounds of chromosome duplication and segregation. Sexual reproduction, on the other hand, relies on formation of haploid gametes from diploid germ cells. This process, known as meiosis, involves two rounds of chromosome segregation following a single round of DNA replication. This remarkable feat is made possible by three key innovations in chromosome behavior. The first is the pairing after their replication of homologous chromosomes and reciprocal recombination between maternal and paternal chromatids. Recombination is initiated by DNA double-strand breaks created by the Spo11 endonuclease (Keeney et al., 1997), whose repair by a homologous chromatid of different parental origin is sometimes accompanied by crossing over. This creates chiasmata (Lee and Orr-Weaver, 2001), which enable sister chromatid cohesion mediated by a meiosis-specific variant of cohesin (containing Rec8 instead of Scc1) to connect maternal chromosomes with their paternal homologs

*Correspondence: nasmyth@nt.imp.univie.ac.at

[4]These authors contributed equally to this work.

(Klein et al., 1999; Parisi et al., 1999). The second innovation is a mechanism that prevents biorientation of sister kinetochores. This is thought to enable Ipl1/Aurora B to promote instead the biorientation of maternal and paternal centromeres; that is, homologous sister centromere pairs are pulled in opposite directions and not sisters themselves.

Only when all homolog pairs come under tension are their chiasmata resolved by the destruction of cohesion between sister chromatids along chromosome arms, due, at least in yeast, to Rec8's cleavage by separase. This triggers anaphase I and the segregation of maternal centromeres from paternal ones (Buonomo et al., 2000). Due to the cosegregation of sister centromeres, meiosis I is often referred to as a reductional division. Meiosis II, during which sister centromeres split (as they do in mitosis), is referred to as an equational division. In reality, meiosis I is equational for sequences distal to chiasmata, whereas meiosis II is equational at centromeres and proximal to chiasmata (Janssens, 1909).

The third innovation of meiotic cells is their ability to protect cohesion in the vicinity of centromeres during the process of chiasmata resolution. Rec8 in the vicinity of centromeres resists separase cleavage, and the cohesion mediated by this population of cohesin complexes makes possible a second round of chromatid segregation (Buonomo et al., 2000; Klein et al., 1999; Watanabe and Nurse, 1999) in the absence of any intervening DNA replication, without which new cohesion cannot be generated (Toth et al., 1999; Uhlmann and Nasmyth, 1998; Watanabe et al., 2001). The ability to resist separase at the onset of anaphase I is a property exclusive to Rec8 in the vicinity of centromeres. Scc1 expressed in its place is capable of conferring cohesion and monoorientation of sister centromeres but is not capable of resisting separase in this region of the chromosome (Toth et al., 2000). Under these circumstances, sister centromeres are drawn to the same pole, but their precocious disjunction prevents biorientation of sister kinetochores during meiosis II.

Though crucial for meiosis I, the mechanism by which biorientiation of sister kinetochores is prevented while that of homologs is promoted is poorly understood. Budding yeast cells produce a meiosis-specific protein called Mam1, whose recruitment to yeast centromeres during meiosis I is essential for eliminating biorientation (Toth et al., 2000). In *mam1*Δ mutants, cells attempt to drag sister centromeres to opposite poles but are prevented from doing so by cohesion between sister centromeres mediated by Rec8. The consequence is chromosome missegregation and gamete aneuploidy. In fission yeast, whose Scc1 subunit (known as Rad21) is also expressed during meiosis and is capable of mediating sister chromatid cohesion (Y. Watanabe, personal communication), the elimination of Rec8 permits not only the biorientation of sister kinetochores but also the disjunction of sister chromatids and results in an equational division (Watanabe and Nurse, 1999).

We describe here two proteins, called Csm1 and Lrs4, which collaborate with Mam1 to promote monoorientation during meiosis I in budding yeast. Neither Csm1 nor Lrs4 are meiosis specific and, surprisingly, they reside as a complex within the nucleolus for most of the yeast life cycle, where they participate in rDNA silencing. They depart from nucleoli only temporarily, shortly before the first meiotic division, whereupon they form a ternary "monopolin" complex with Mam1, which binds to centromeres. Csm1 is the most conserved of monopolin's three constituents and its *S. pombe* homolog (Pcs1) also resides within nucleoli and centromeres. Surprisingly, Pcs1 has a role in kinetochore function in mitosis and meiosis II but little if any role in meiosis I. We suggest that monopolin and Pcs1 may have a common activity, namely, clamping together microtubule attachment sites, be they on the same or different chromatids.

Results

A Selection Scheme for Monopolin Mutants

Because Mam1's sequence is poorly conserved and provides no clue as to its function, we set out to identify further factors. To do this, we identified a strain that produces dead spores when monopolin is active but viable ones when inactive. Cells lacking *SPO12* form asci containing two spores (dyads) of intermediate viability (Klapholz and Esposito, 1980). They undergo only a single division, in which at least some chromosomes are segregated reductionally (Sharon and Simchen, 1990). Deletion of *SPO11* in this background causes all spores to be inviable (Figure 1A). We suspected that the inviability of *spo11*Δ *spo12*Δ dyads was due to cosegregation of sister centromeres without biorientation of homologs. If so, deletion of *MAM1* should cause sister centromeres to segregate equationally and produce viable spores. Remarkably, this is indeed the case (Figure 1A and data not shown).

To identify additional genes required for monopolin function, we looked for new mutations capable of causing a haploid *spo11*Δ *spo12*Δ strain (carrying a copy of both mating types) to produce viable spores. The parental strain was mutagenized with a library of gene disruptions created by a bacterial transposon. Mutants derived from disruptions in specific genes were identified by sequencing PCR products generated from the insertion sites. The genes thereby identified included *MAM1* (seven times) as well as others not previously directly implicated in monopolin function, such as *SPO13* (four times), *CSM1* (24 times), *CDC55* (twice, though neither was null), *PPM1* (once), and *ASE1* (once). Disruption of *SPO13* has pleiotropic consequences and it must, therefore, have several meiotic functions besides possibly promoting monopolin activity (Klapholz and Esposito, 1980; our unpublished observations). Disruption of *ASE1* (which encodes a protein associated with anaphase spindles) or *PPM1* (a PP2A methyl transferase) had only minor effect on meiotic chromosome segregation in an otherwise wild-type background. Full deletion of *CDC55* (a regulatory subunit of PP2A) so severely reduced sporulation that it was not possible to assess its effect on chromosome segregation.

Chromosome Segregation during Meiosis Requires Csm1 and Lrs4, Nucleolar Proteins Involved in rDNA Silencing

CSM1 (chromosome segregation in meiosis; ORF YCR086W) has already been shown to have a role in meiotic chromosome segregation (Rabitsch et al., 2001).

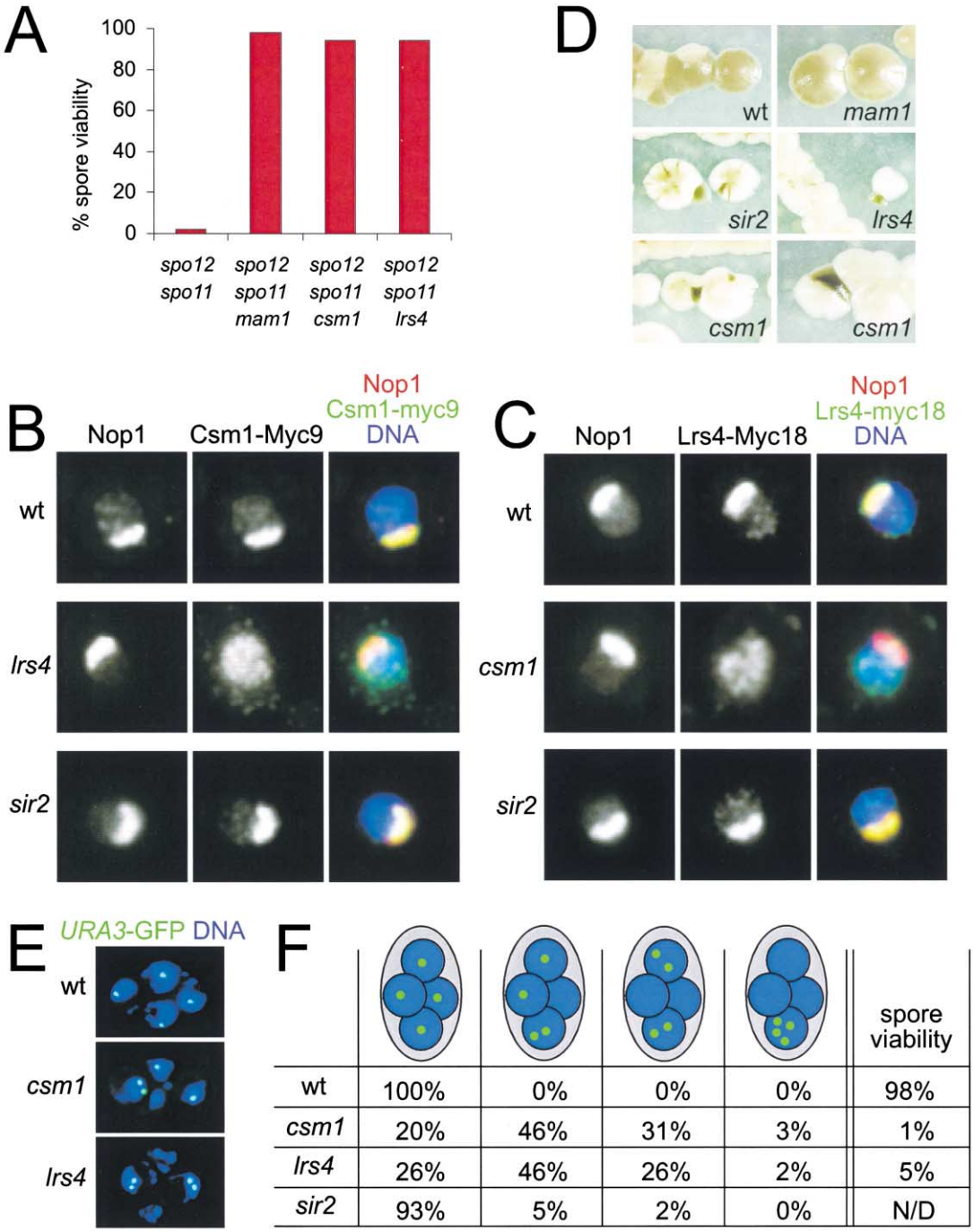

Figure 1. Csm1 and Lrs4 Are Nucleolar Proteins Required for Chromosome Segregation during Meiosis

(A) *spo12*Δ *spo11*Δ (strain K9277), *spo12*Δ *spo11*Δ *mam1*Δ (K9278), *spo12*Δ *spo11*Δ *csm1*Δ (K10016), and *spo12*Δ *spo11*Δ *lrs4*Δ (K10009) were sporulated on plate and dyads were dissected on YEPD and grown for 2 days (n = 100 spores) at 30°C.

(B and C) Wild-type (K9466), *lrs4*Δ (K9971), and *sir2*Δ (K9970) cells expressing Csm1-Myc9 (B) and wild-type (K10139), *csm1*Δ (K10235), and *sir2*Δ (10217) cells expressing Lrs4-Myc18 (C) were grown to log phase in 2% YPA, and fixed and stained with antibodies against Myc and Nop1.

(D) Wild-type (JS306), *mam1*Δ (K10049), *sir2*Δ (K10050), *lrs4*Δ (JS575), and two clones of *csm1*Δ (K10052 and 10053), which all harbored a *RDN1::Ty1-MET15* construct (Smith et al., 1999), where grown on MLA plates for 5 days. Expression of *MET15* at wild-type levels causes a light brown colony color. White colony color is an indication of elevated expression and black sectors indicate derepression of mitotic recombination in the rDNA.

(E and F) Segregation of the *URA3* locus (which was marked on both homologs by GFP) in wild-type (K8409), *csm1*Δ (K10312), *lrs4*Δ (K9928), and *sir2*Δ (K9972) tetrads. Shown are sample pictures (E) and quantification (n = 200) of different classes of tetrads (F). In many tetrads, fewer than four individual GFP signals were visible; in these cases, we used the number of spores lacking a signal as our classification criterion.

We confirmed that its disruption, in an otherwise wild-type background, caused a very similar pattern of chromosome missegregation to that caused by deletion of *MAM1*. The resulting spores contain unequal amounts of DNA, some with no copies and others with several copies of a GFP-marked (Michaelis et al., 1997) chromosome V (Figure 1F). *CSM1* is expressed in vegetative as well as meiotic cells, and its deletion causes a growth defect in glucose but not acetate medium. The former is not due to chromosome missegregation during mitosis because deletion of *CSM1* in a strain carrying a supernumary chromosome III (Spencer et al., 1990), whose loss can be measured by a colony color assay, had no effect on the rate of chromosome loss (data not shown).

To determine Csm1p's location within the cell, we tagged the gene with nine Myc epitopes. Surprisingly, Csm1-Myc9 (which was partially functional; spores from a homozygous diploid are approximately 50% viable) largely colocalized with the nucleolar protein (Aris and Blobel, 1988) Nop1 (Figure 1B, upper panel). Genome-wide two-hybrid data (Uetz et al., 2000) raised the possibility that Csm1 might interact with a protein called Lrs4 (loss of rDNA silencing; product of ORF YDR439W) that had been implicated in rDNA silencing (Smith et al., 1999). Though we did not pick up *lrs4*Δ mutations in our screen, deletion of *LRS4* also fully rescued the spore viability of *spo11*Δ *spo12*Δ double mutants (Figure 1A) and caused *csm1*Δ-like chromosome missegregation and low spore viability during meiosis when deleted in otherwise wild-type cells (Figures 1E and 1F). It also caused a growth defect similar to that caused by *csm1*Δ, but no increase in chromosome loss during mitosis (data not shown). Myc18-tagged Lrs4 protein (which was fully functional) also colocalized with Nop1 in the nucleolus (Figure 1C, upper panel).

Csm1-Myc9 and Lrs4-Myc18 no longer colocalized with Nop1 in *lrs4*Δ and *csm1*Δ mutants, respectively, although the distribution of Nop1 was unaltered by either mutation. In the absence of the other protein, Csm1-Myc9 and Lrs4-Myc18 were found throughout the nucleus and partly also in the cytoplasm (Figures 1B and 1C, middle panels). Deletion of *SIR2*, which has an even more crucial role in rDNA silencing than *LRS4* (Smith et al., 1999), had little deleterious effect on meiotic chromosome segregation (Figure 1F) or on the localization of Csm1-Myc9 or Lrs4-Myc18 proteins (Figures 1B and 1C, lower panels). Therefore, rDNA silencing per se cannot be required either for monopolin function or for Csm1 and Lrs4 nucleolar localization. Csm1 and Lrs4 must have very specific roles in each other's recruitment to the nucleolus. Deletion of *CSM1*, but not *MAM1* (which is not at all expressed in mitotic cells) caused a similarly modest loss of silencing of *MET15* (Figure 1D) and *URA3* (not shown) reporter genes located within the rDNA array as that of *LRS4*.

CSM1 and *LRS4* Are Required for the First Meiotic Nuclear Division

To investigate the cause of chromosome missegregation in *csm1*Δ and *lrs4*Δ mutants, we compared their meiotic progression with wild-type cells (Figure 2A). To facilitate our analysis, Pds1 was tagged with 18 Myc epitopes, and one of the two parental chromosome Vs was marked by GFP tethered at the *URA3* locus situated 30 kb from the centromere. Both premeiotic DNA replication (as measured by FACS) and recombination at the *LEU2* hotspot (as measured by Southern blotting; only for *csm1*Δ) were similar if not identical to wild-type, as was the formation and disassembly of synaptonemal complex during pachytene (data not shown).

The first sign of any abnormality occurred when cells tried to segregate chromosomes at the onset of anaphase I. In wild-type cells, destruction of Pds1 is immediately followed by chiasmata resolution, which triggers the division of DAPI-staining material into two equal masses (nuclear division), bipolar spindle elongation, and the cosegregation of sister centromeres to one pole (Figure 2B, upper panel). Sister centromeres (*URA3*-GFP) only segregate to opposite poles after a second round of Pds1 destruction during anaphase II (Figure 2A, green triangles versus pink squares and Figure 2B, upper panel). In *csm1*Δ or *lrs4*Δ mutants, nuclei did not divide and spindles elongated only modestly following Pds1's disappearance, causing the abnormal accumulation of Pds1-negative cells with metaphase-like spindles. Strikingly, sister centromere (*URA3*) sequences frequently split along the spindle axis in mononucleate Pds1-negative mutant cells (Figure 2B, middle and lower panels), and clearly did so earlier than any nuclear division (Figure 2A, green triangles versus blue diamonds). Both *csm1*Δ and *lrs4*Δ mutants subsequently undergo a highly abnormal meiotic division at the same time as the second meiotic division in wild-type (Figure 2A, pink squares in left graph versus blue diamonds in middle and right graphs). During this division, one DNA mass is segregated to four spindle poles, resulting in four unequally sized nuclei. Presumably due to the multipolar nature of the division, sister *URA3* sequences frequently segregated on different spindle axes (Figure 2B, middle and bottom panels). These phenotypes of *csm1*Δ and *lrs4*Δ mutants resemble closely those of *mam1*Δ mutants.

Expression of the meiosis-specific cohesin subunit Rec8 was normal in *csm1*Δ and *lrs4*Δ mutants on immunoblots (data not shown). Furthermore, the kinetics of appearance and disappearance of chromosome spreads lacking bulk Rec8 (Figure 2C, red squares), with Rec8 only at centromeres (Figure 2C, brown triangles), and lacking Rec8 from the entire chromatin were unaffected in *csm1*Δ and *lrs4*Δ as compared to wild-type cells. Whereas chromosome spreads with centromere-only Rec8 were binucleate in wild-type, they were invariably uninucleate in *csm1*Δ and *lrs4*Δ mutants (Figure 2D), though the DNA was frequently stretched. This confirms that they had indeed failed to undergo nuclear division following cleavage of Rec8 along chromosome arms. A corollary is that the lack of nuclear division in *csm1*Δ and *lrs4*Δ mutant cells is unlikely to be due to a failure of separase to destroy arm cohesion.

Replacement of Rec8 by Scc1 Permits *csm1*Δ and *lrs4*Δ Mutants to Undergo a Fully Equational Meiosis I Division

The persistence of Rec8 at centromeres in *csm1*Δ and *lrs4*Δ mutants suggests that the splitting of sister *URA3* sequences in cells that have just destroyed Pds1 is unlikely to be caused by a precocious loss of cohesion.

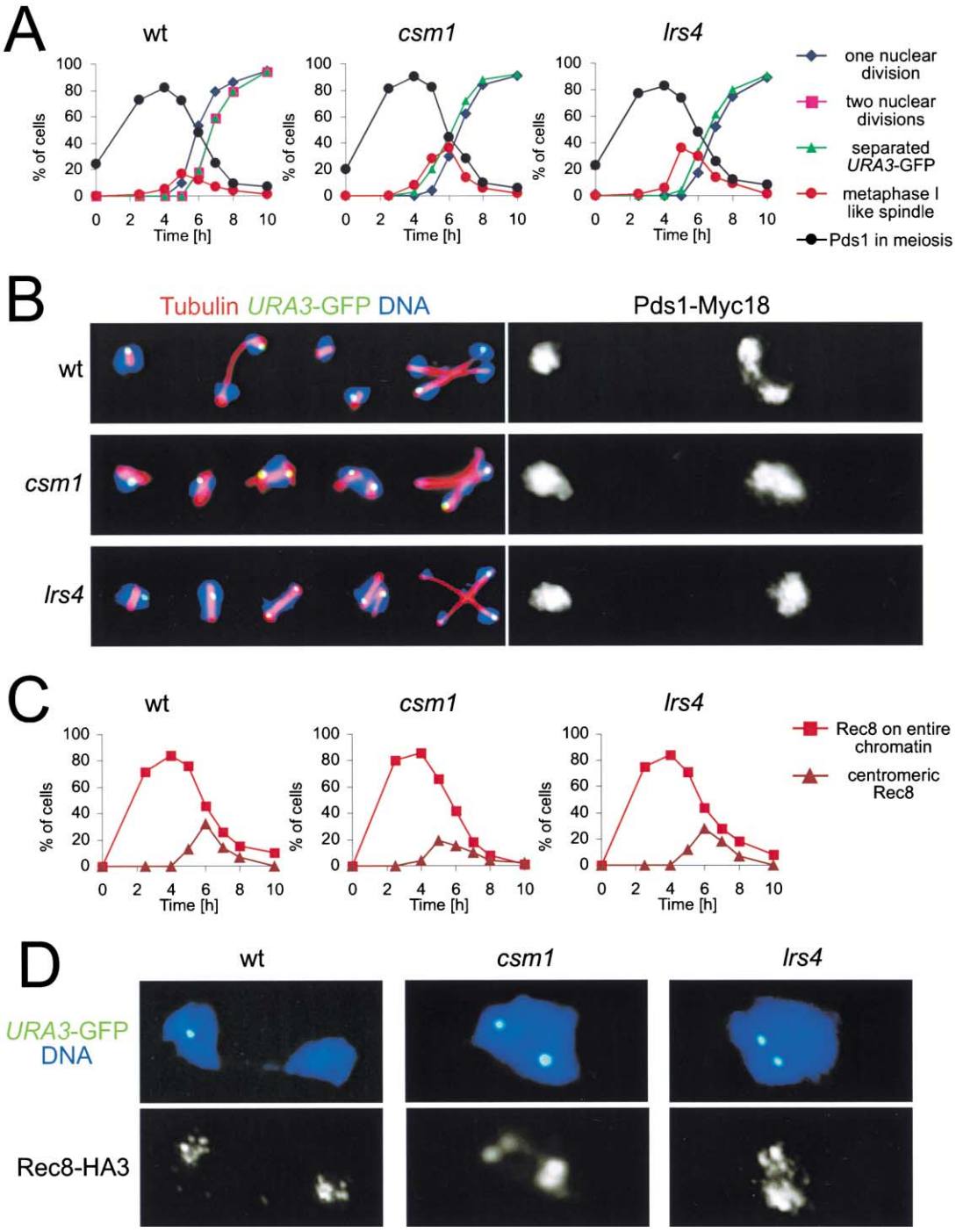

Figure 2. *CSM1* and *LRS4* Are Required for the First Meiotic Nuclear Division

(A) Progression of wild-type (K10003), *csm1*Δ (K9797), and *lrs4*Δ (K10004) strains with the *URA3* locus of one homolog marked by GFP and expressing Rec8-HA3 and Pds1-Myc18 through meiosis in liquid culture. At indicated time points, immunofluorescence was performed. Shown are fractions of cells that have undergone at least one nuclear division (blue diamonds), a second division (pink squares), that contain a short bipolar spindle (red circles), that have separated sister *URA3* sequences (green triangles), and that contain Pds1 in the nucleus (black circles).
(B) Composite images of cells from the same experiment as in (A) showing progression through meiosis from left to right.
(C) Chromatin spreads of cells from the same experiment as in (A) were prepared and stained with antibodies against HA. The accumulation of spreads that contain Rec8 all over the chromatin (red squares) or Rec8 confined to one or two clusters (brown triangles) is shown.
(D) Sample chromatin spreads from (C), which show staining for Rec8 confined to one or two clusters.

We propose that Csm1 and Lrs4 are, like Mam1, essential for monoorientation of sister kinetochores but not for protecting centromeric Rec8 during meiosis I. Mutants defective in any one of these three proteins attempt to pull sister centromeres in opposite directions at the first meiotic division but are prevented from performing this

successfully by cohesin that has resisted separase cleavage in the vicinity of centromeres. The frequent sister centromere splitting that occurs in these mutants may be caused by cohesion at centromeres being overwhelmed by spindle forces tending to split sisters during a prolonged metaphase-like state.

If this hypothesis is correct, then replacement of Rec8 by Scc1 (*rec8Δ::SCC1*), which cannot survive separase activation at centromeres, should permit *csm1Δ* and *lrs4Δ* mutants to undergo a fully equational meiosis I division. Because Rec8 is required for recombinogenic meiotic double-strand break repair and Scc1 cannot substitute for this function (Toth et al., 2000), it is necessary to perform these experiments in cells lacking Spo11. Deletion of *SPO11* abolishes chiasmata, leaving homologs unconnected, but, due to monopolin, sister centromeres are dragged to the same pole. As a consequence, the first meiotic division can take place without separase activation (i.e., before Pds1 destruction) in *spo11Δ* cells. Cells containing metaphase I spindles are very rare (compare wild-type in Figure 2A with *spo11Δ* in Figure 3C, red circles), and many anaphase I cells possess high levels of Pds1 (data not shown). The same is true of *spo11Δ rec8Δ::SCC1* double mutants (Figure 3A, left graph and Figure 3B, left panel). Deletion of *CSM1* or *LRS4* in *spo11Δ rec8Δ::SCC1* cells abolished nuclear division in the presence of Pds1 (Figure 3B, middle and right panels) and increased the number of cells with metaphase I spindles (Figure 3A, middle and right graphs, red circles) to levels seen in wild-type (Figure 2A, left graph, red circles) but lower than *csm1Δ* or *lrs4Δ* single mutants (Figure 2A, middle and right graphs, red circles) or *spo11Δ csm1Δ* or *spo11Δ lrs4Δ* double mutants (Figure 3C, middle and right graphs, red circles). Even more striking, *csm1Δ spo11Δ rec8Δ::SCC1* and *lrs4Δ spo11Δ rec8Δ::SCC1* triple mutants underwent a nuclear division as soon as Pds1 disappeared from the cells, and sister chromatids were invariably segregated to opposite poles (Figures 3A and 3B). The meiotic program, nevertheless, rolls on, and a second division during which sister centromeres segregate at random (data not shown) follows.

The rescue of nuclear division (by *rec8Δ::SCC1* replacement in *spo11Δ csm1Δ* or *spo11Δ lrs4Δ* cells) and the conversion of reductional to equational chromosome segregation (by deletion of either *CSM1* or *LRS4* in *spo11Δ rec8Δ::SCC1* cells) during meiosis I was not due simply to deletion of *SPO11*, because *spo11Δ csm1Δ* and *spo11Δ lrs4Δ* double mutants failed to undergo nuclear division at meiosis I, accumulated cells with metaphase I-like spindles (many without Pds1), and frequently separated sister centromere sequences before nuclear division occurred (Figure 3C). We therefore conclude that Rec8, which can survive separase at centromeres, but not Scc1, which cannot, prevents *csm1Δ* or *lrs4Δ* mutants from undergoing a meiosis I division. This confirms that Csm1 and Lrs4 are, together with Mam1, needed for ensuring monoorientation of sister centromeres but not for protecting their cohesion from separase.

Csm1 and Lrs4 Relocalize from the Nucleolus to Centromeres Transiently during Meiosis I

To investigate how proteins expressed during mitosis and localized in the nucleolus could regulate kinetochore function, we investigated the localization of Csm1 and Lrs4 during meiosis, during which they are both constitutively expressed (data not shown). In fixed cells, both Myc-tagged proteins colocalized with Nop1 from the transfer of cells into sporulation medium until late prophase (Figure 4A, left-hand cells). Strikingly, when cells had progressed to metaphase I and formed bipolar spindles they were found throughout the nucleus and were no longer concentrated in the nucleolus (Figure 4A, cells second from left). This delocalization is only transient because both proteins were again concentrated within the as yet undivided nucleoli of anaphase I cells (Figure 4A, cells second from right). Neither protein departed from the nucleoli of metaphase II cells (Figure 4A, right-hand cells) or of mitotic metaphase cells (data not shown).

Because individual kinetochores cannot be visualized in fixed cells, we scrutinized Csm1-Myc9 and Lrs4-Myc18 localization on chromatin spreads. From the transfer to sporulation medium and through early prophase stages (spread nuclei were staged using staining for the synaptonemal complex protein Zip1), Csm1 and Lrs4 were found tightly associated with the nucleolus (data not shown). The situation changes in pachytene, when in 6/15 nuclei for Csm1 and 4/15 nuclei for Lrs4, additional foci (more than seven) that colocalized with Ndc10 (a kinetochore component; Kitagawa and Hieter, 2001) tagged with six HA epitopes appeared (Figure 4C, upper panel). During late stages of pachytene, when Zip1 appears patchy, the subset of nuclei that showed clear colocalization between Csm1 or Lrs4 and Ndc10 (more than seven foci) increased (8/15 nuclei for Csm1 and 10/15 nuclei for Lrs4; Figure 4B, upper panel). After desynapsis was complete but before nuclear division, Csm1 and Lrs4 foci were frequently found as pairs colocalizing with pairs of Ndc10 foci, which presumably represent separated but close homologous centromeres (Figures 4B and 4C, lower panels). These observations suggest that Csm1 and Lrs4 start to localize to the vicinity of centromeres at late pachytene or around the same time as they depart from nucleoli. Csm1 and Lrs4 are, therefore, found at centromeres during the period in which they prevent biorientation of sister kinetochores. A similar timing of localization to centromeres has been reported for Mam1 (Toth et al., 2000).

We also investigated whether Mam1, Csm1, and Lrs4 bind to kinetochores in the absence of each other. Mam1's association with kinetochores was drastically reduced in *csm1Δ* or *lrs4Δ* cells, as was Csm1 and Lrs4's association in *mam1Δ* cells. As in the case of their nucleolar localization, association of Csm1 and Lrs4 with kinetochores was interdependent. However, the release of Csm1 and Lrs4 from nucleoli was unaffected by deletion of *MAM1* (see Supplemental Figure S1, available at http://www.developmentalcell.com/cgi/content/full/4/4/535/DC1, and the accompanying text).

Csm1, Lrs4, and Mam1 Form a Trimeric Complex

Cytological evidence as well as the identical phenotypes of *csm1Δ* and *lrs4Δ* mutants suggest an intimate relationship between Csm1 and Lrs4. To examine whether they form a complex together, we constructed a strain that expressed Myc9-tagged Csm1 and HA3-tagged Lrs4 proteins from their endogenous loci. Lrs4-HA3 (Figure 5A, lane 4, middle panel) but not the outer nucleolar

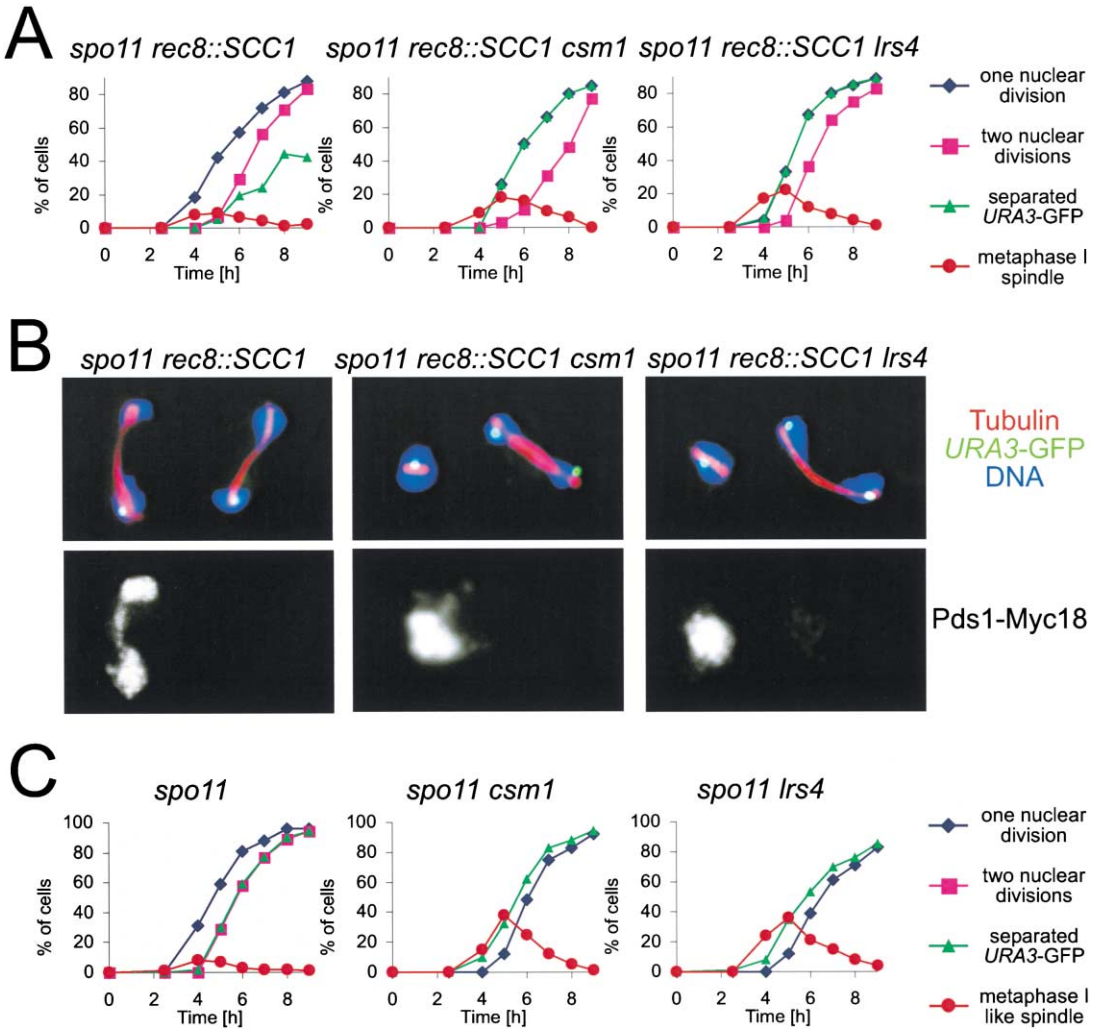

Figure 3. *CSM1* and *LRS4* Are Required for Monoorientation of Sister Kinetochores in Meiosis I

(A) Progression of *spo11*Δ *rec8*Δ*::SCC1* (K10512), *spo11*Δ *rec8*Δ*::SCC1 csm1*Δ (K9694), and *spo11*Δ *rec8*Δ*::SCC1 lrs4*Δ (K10515) strains with the *URA3* locus of one homolog marked by GFP and expressing Rec8-HA3 and Pds1-Myc18 through meiosis in liquid culture. At indicated time points, immunofluorescence was performed. Shown are the fraction of cells that have undergone at least one nuclear division (blue diamonds), a second division (pink squares), that contain a short bipolar spindle (red circles), and that have separated sister *URA3* sequences (green triangles).

(B) Sample images of cells from the same experiment as in (A).

(C) Progression of *spo11*Δ (K10506), *spo11*Δ *csm1*Δ (K9901), and *spo11*Δ *lrs4*Δ (K10509) strains with the *URA3* locus of one homolog marked by GFP and expressing Rec8-HA3 and Pds1-Myc18 through meiosis in liquid culture. The experiment was carried out as in (A).

protein Nop1 (Figure 5A, lane 4, lower panel) efficiently coimmunoprecipitated with Csm1-Myc9 using anti-Myc antibodies (Figure 6A, lane 4, upper panel) from extracts of cycling vegetative cells. Coimmunoprecipitation was dependent on the presence of the Myc tag on Csm1 (Figure 5A, lane 2, middle panel). Conversely, Csm1-Myc9 but not Nop1 efficiently coimmunoprecipitated with Lrs4-HA3 when the latter was immunoprecipitated with anti-HA antibodies (data not shown).

Both Csm1 and Lrs4 proteins contain sequences predicted to form coiled-coil structures (amino acids 31–70 in Csm1 and 46–78 plus 98–118 in Lrs4; Lupas et al., 1991). To test whether Csm1 and Lrs4 bind to each other via these sequences, we measured the binding of in vitro-translated Lrs4 to bacterially expressed and purified full-length and truncated Csm1 proteins fused to maltose binding protein (MBP). Lrs4 bound efficiently to a full-length MBP-Csm1 matrix but not at all to a control matrix containing only MBP (Figure 5B, lanes 2 and 3). Lrs4 still bound to an MBP-Csm1 fusion lacking all but Csm1's N-terminal coiled-coil region (Figure 5B, lanes 4 and 5). It did not bind to fusions containing the rest of the Csm1 protein (Figure 5B, lanes 6 and 7). We also tested the binding of in vitro-translated Csm1 to full-length and truncated versions of Lrs4 fused to MBP. Both of Lrs4's predicted coiled-coil stretches were required for Csm1 binding (Figure 5C, lanes 4 and 7). This region along with the N terminus was sufficient for binding (Figure 5C, lanes 5 and 6).

To investigate whether Csm1 and Lrs4 form a trimeric complex with Mam1, we tested whether in vitro-translated Csm1 or Lrs4 can alone or together bind to a matrix

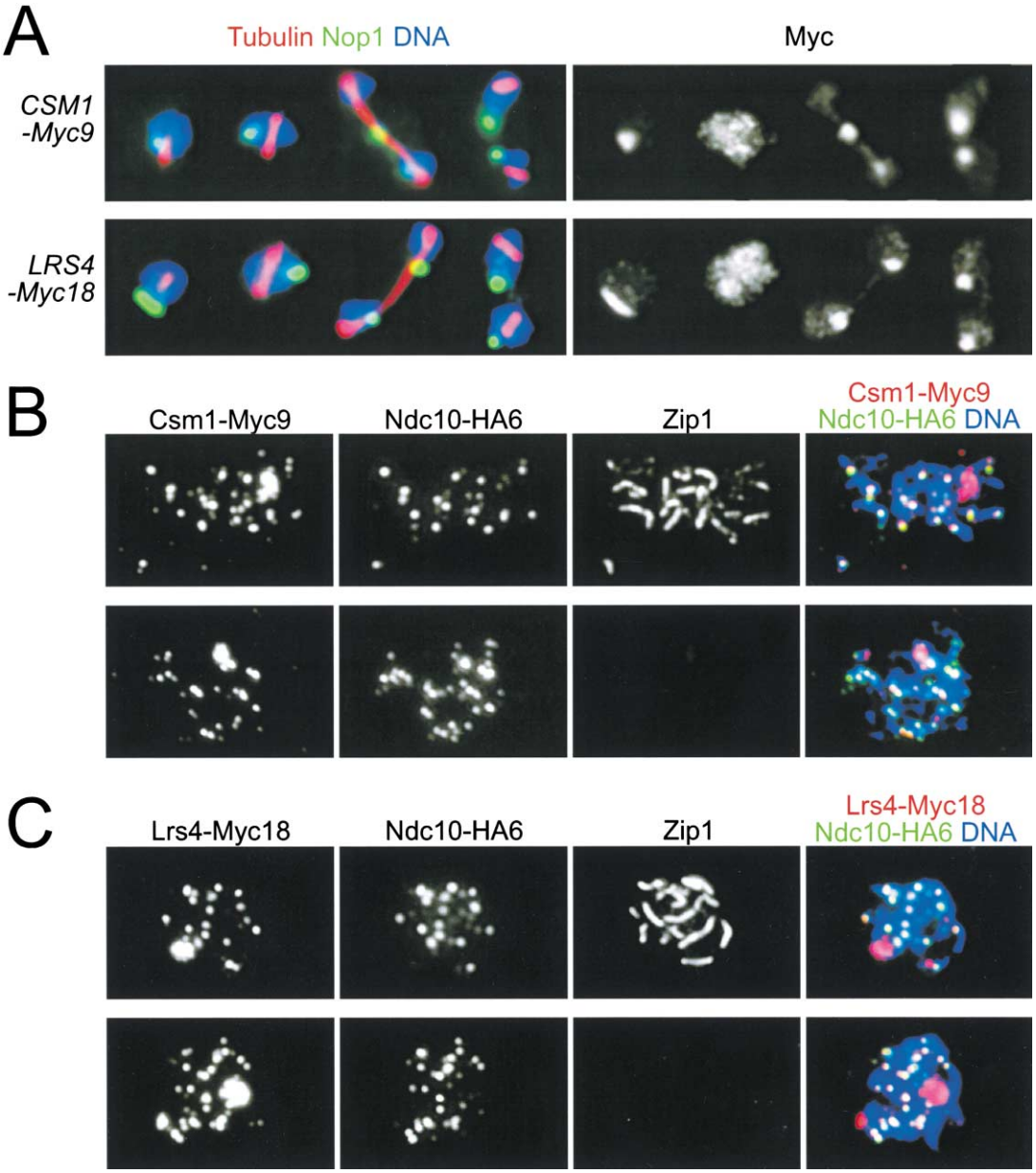

Figure 4. Csm1 and Lrs4 Are Transiently Released from the Nucleolus and Localize to Centromere Regions during Meiosis I

(A) Cells expressing Ndc10-HA6 and Csm1-Myc9 (K9118) or Lrs4-Myc18 (K10418) were sporulated and immunofluorescence was performed. (B and C) Chromatin spreads of cells from the same experiment as in (A) were stained with antibodies against Myc, HA, and Zip1.

containing MBP-Mam1. Csm1 bound efficiently to MBP-Mam1 even in the absence of Lrs4 (Figure 5E, lane 6), but Lrs4 was only able to bind in the presence of Csm1 (Figure 5E, lane 7 versus lane 8). The C-terminal 122 amino acids of Mam1 were sufficient for Csm1 binding (Figure 5D, lane 11). These data are consistent with genome-wide two-hybrid assays (Uetz et al., 2000) and suggest that Csm1 and Lrs4 bind to each other via their coiled-coil domains and integrate Mam1 during meiosis I by virtue of the interaction between Csm1 and the C-terminal half of Mam1 (Figure 5F).

Csm1, Lrs4, and Mam1 Associate with *CEN* DNA

Monopolin could either bind sister kinetochores together so that they act as a single unit or it could modify the surrounding chromatin in a manner that was incompatible with the biorientation process. This raises the question whether monopolin acts only at sites of microtubule attachment, which in budding yeast are confined to about 200 base pairs (known as *CEN*), or on a wider region surrounding this. We therefore investigated whether Csm1, Lrs4, or Mam1 can be crosslinked by formaldehyde to DNA in the vicinity of centromeres and if so, to what sequences.

Meiotic cells expressing Myc-tagged monopolin components along with HA-tagged Ndc10 were treated with formaldehyde 6 hr after transfer to sporulation medium and then sonicated to shear DNA to about 1 kbp fragments. The amounts of specific DNA fragments precipitated by Myc- (Myc ChIP) or HA- (HA ChIP) specific

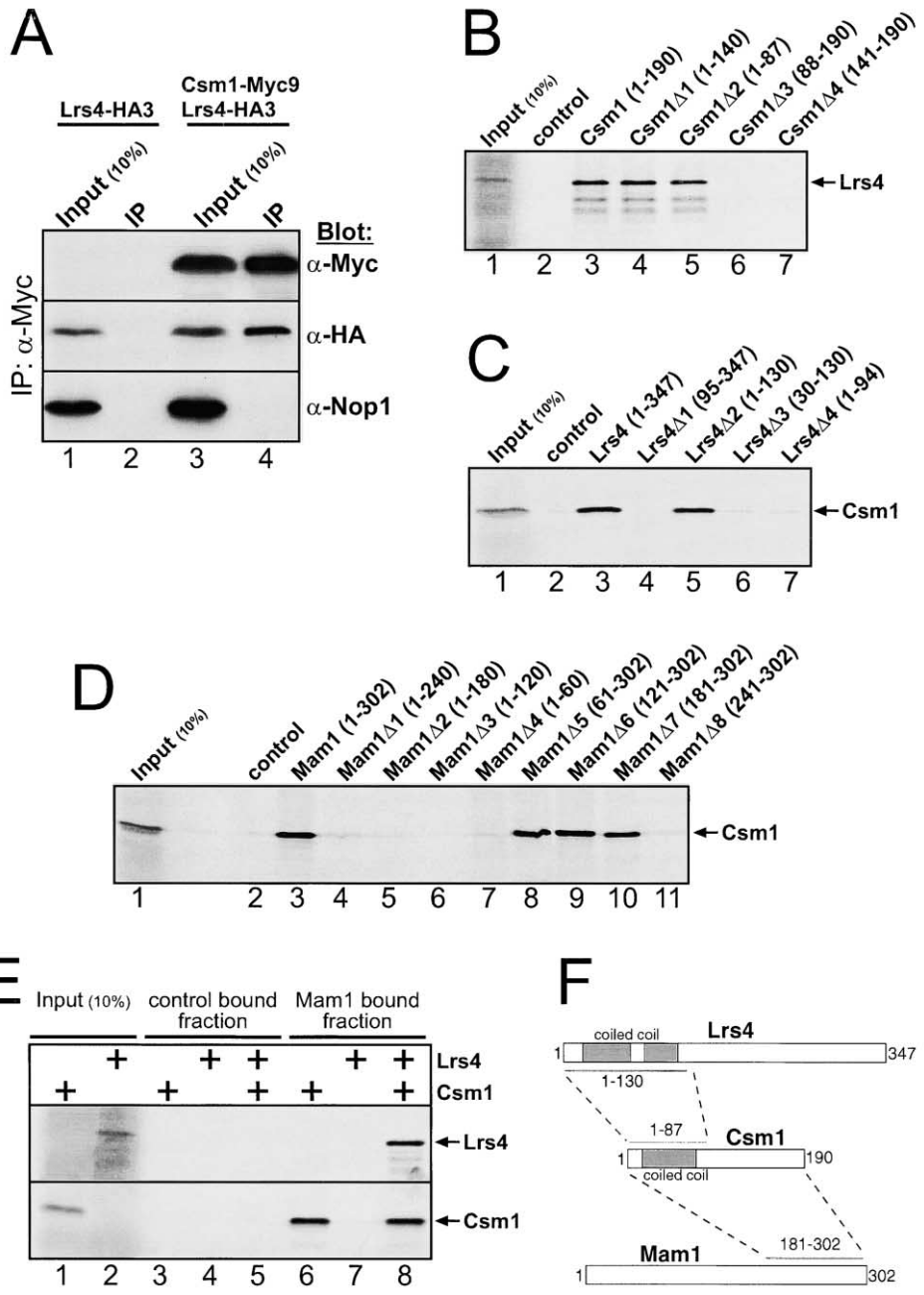

Figure 5. Mam1, Csm1, and Lrs4 Form a Ternary Monopolin Complex

(A) Coimmunoprecipitation of Csm1 and Lrs4: protein extracts from mitotic cultures of strains expressing Lrs4-HA3 alone (K10249) and Csm1-Myc9 and Lrs4-HA3 together (K10253) were immunoprecipitated using an anti-Myc antibody. The extract (input lanes 1 and 3) and the precipitates (IP lanes 2 and 4) were subsequently analyzed by immunoblotting.

(B–D) In vitro binding assays: maltose binding protein (MBP) fusion proteins of Csm1 (B), Lrs4 (C), and Mam1 (D) and various deletion variants thereof were immobilized on an amylose resin, incubated with in vitro-translated [^{35}S] radioactively labeled Lrs4 (B) or Csm1 (C and D), and precipitated. The input (lane 1) and fractions of the labeled proteins bound to the MBP fusion protein-coupled resins (other lanes) were analyzed by SDS-PAGE and phosphor imaging. The in vitro-translated proteins were incubated with MBP alone as a control.

(E) In vitro reconstitution of the ternary Mam1-Csm1-Lrs4 complex: in vitro-translated and radioactively labeled Csm1 (lanes 3 and 6), Lrs4 (lanes 4 and 7), and both Csm1 and Lrs4 together (lanes 5 and 8) were incubated with either MBP as a control (lanes 3–5) or an MBP-Mam1 fusion protein immobilized on an amylose resin (lanes 6–8) and precipitated. The input (lanes 1 and 2) and the fractions of the labeled proteins bound to the resin (lanes 3–8) were analyzed by SDS-PAGE and phosphor imaging. In all binding studies (A–E), 10% of the input used for the binding experiment is loaded in the input lane.

(F) Molecular architecture of the monopolin complex: Lrs4 and Csm1 interact directly via their N-terminal coiled-coil domains. Csm1 binds to the C terminus of Mam1, linking Lrs4 to Mam1. The gray boxes indicate regions within Lrs4 and Csm1 with a high coiled-coil prediction. The dashed lines and gray bars indicate the determined interaction domains between the monopolin complex subunits.

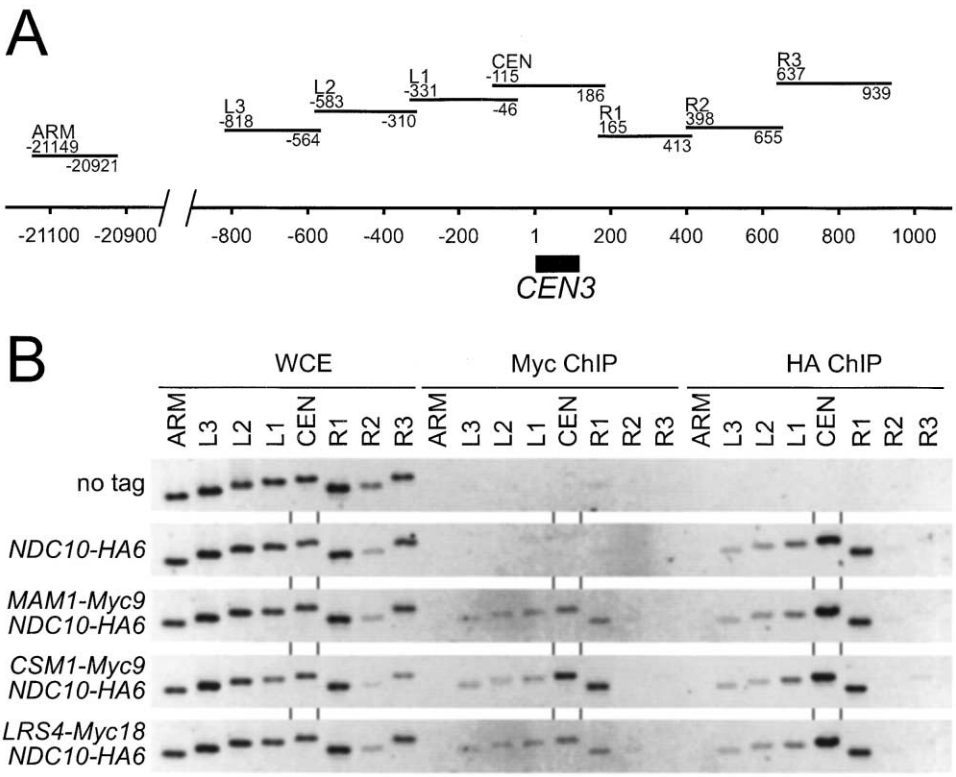

Figure 6. Mam1, Csm1, and Lrs4 Associate with *CEN*3 DNA

(A) Position of primer pairs relative to *CEN3* on chromosome III used for ChIP analysis of monopolin association with chromatin around centromeres in (B). Indicated are the positions of oligonucleotides' 5′ ends relative to base 1 of *CEN3* and the primer pairs' names.

(B) PCR analysis of coprecipitated sequences with antibodies against Myc and HA in strains expressing no tagged protein (K10103), Ndc10-HA6 (K9692), or Ndc10-HA6 and Mam1-Myc9 (K9962), Csm1-Myc9 (K9118), or Lrs4-Myc18 (K10418). WCE lanes indicate PCR products generated from whole-cell extract for reference and Myc- and HA-labeled lanes show analysis of anti-Myc and anti-HA immunoprecipitates, respectively. The percentage of cells in metaphase I was measured by immunofluorescence on fixed cells: 20% of K10103, 21% of K9692, 16% of K9962, 21% of K9118, and 19% of K10418 cells.

antibodies were measured by PCR using primer pairs situated in the vicinity of the chromosome III centromere (Figure 6). These were compared to the amount of the same sequences in the starting lysates (WCE; whole-cell extract). With HA antibodies, *CEN3* itself, and to a progressively lesser extent sequences to its left (L1–L3) and right (R1–R3), coprecipitated with Ndc10-HA6 but not with untagged Ndc10. A similar pattern was found in immunocoprecipitates from Mam1-Myc9, Csm1-Myc9, and Lrs4-Myc18 cells but not in untagged cells using Myc antibodies. These data suggest that monopolin associates with and acts at centromeres themselves.

A Protein Homologous to Csm1 (Pcs1) Is Located at Centromeres and Nucleoli in *S. pombe* and Is Involved in Chromosome Segregation

We identified in genome databases Csm1-like proteins from *S. pombe*, *Neurospora crassa*, and *Candida albicans* (Figures 7A and 7B) and other ascomycota (data not shown). Mam1 is less conserved and Lrs4 even less so. We found Mam1-related proteins in *Ashbya* and *Kluyveromyces* genera but Lrs4-like proteins only among *Saccharomyces* yeasts (data not shown).

The putative *S. pombe* Csm1 homolog, which we call Pcs1 (*pombe* chromosome segregation; ORF SPAC11E3.03), shares 22% sequence identity over 189 amino acids with Csm1 and contains predicted coiled-coil stretches (Lupas et al., 1991) in its amino terminus (amino acids 50–74 and 91–130). To investigate its cellular distribution, the endogenous *pcs1* gene was fused to GFP. Pcs1-GFP was concentrated within nucleoli during G2 in mitotic cells (Figure 7C). Some fluorescence was also found at the nuclear periphery that colocalized with an HA-tagged version of the centromere proteins Mis6 (Saitoh et al., 1997) or Bub1 (Bernard et al., 1998), throughout most if not all stages of vegetative growth (Supplemental Figures S2A and S2B, and data not shown) and meiotic development apart from the horse tail stage of prophase I (Supplemental Figures S2C and S2D, and data not shown).

Strains lacking the *pcs1* gene are viable but have a high frequency of lagging chromosomes in late anaphase cells (Figure 7E; 28% in Δ*pcs1* cells compared to 0% in wild-type), which is associated with a high rate of sister chromatid nondisjunction. Sister *cen1* sequences marked by GFP (Nabeshima et al., 1998) segregated to the same pole in 7.1% of Δ*pcs1* cells but never

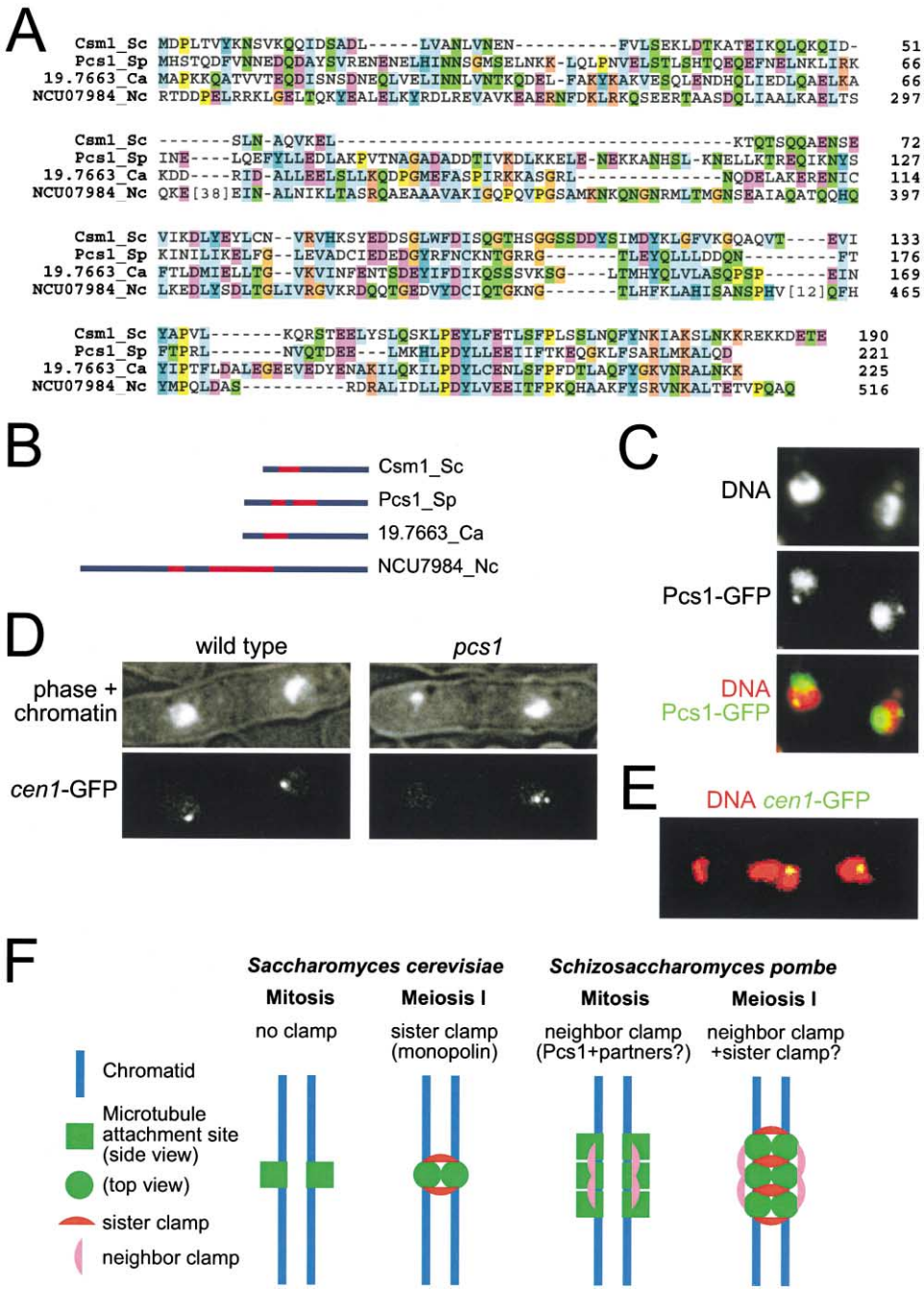

Figure 7. Csm1 Is Conserved in Fungi and Its *S. pombe* Homolog Shares Csm1's Localization but Is Required for Mitosis

(A) Multiple sequence alignment of Csm1 from *Saccharomyces cerevisiae* (Csm1_Sc) and Pcs1 from *Schizosaccharomyces pombe* (Pcs1_Sp), and translations of ORF 19.7663 from *Candida albicans* (19.7663_Ca) and ORF NCU07984 from *Neurospora crassa* (NCU07984_Nc).

(B) Schematic maps of the same proteins as in (A) indicating predicted (Lupas et al., 1991) coiled-coil regions in red.

(C) Pcs1 localizes to the centromere cluster and nucleolus of *S. pombe* cells. Unfixed Pcs1-GFP-expressing G2 cells (strain JP1227) were stained with Hoechst and examined under the fluorescence microscope.

(D) Segregation of chromosome I marked by GFP at *cen1* in wild-type (JP1345) and Δ*pcs1* (JP1262) cells. Unfixed exponentially growing cells were stained with Hoechst and examined under the fluorescence microscope.

(E) Lagging chromosomes are single chromatids in Δ*pcs1* cells. Shown is a sample unfixed anaphase cell (strain 1262) stained with Hoechst that has *cen1* marked with GFP.

(F) A model suggesting a common biological function for monopolin in *S. cerevisiae* and Pcs1 in *S. pombe*, namely clamping microtubule attachment sites.

in wild-type (Figure 7D). A premature loss of sister chromatid cohesion is unlikely to be the cause of these lagging chromosomes, because deletion of *pcs1* caused only a modest increase (from 5.8% to 11.2%) in the separation of DNA sequences proximal to centromere 1 in cells arrested in metaphase due to inactivation of the APC/C by a *cut9* mutation. Deletion of *swi6*, which abolishes cohesin enrichment at centromeres, increases this value to 80% (Bernard et al., 2001). A failure to resolve sister chromatid cohesion on time is also unlikely to be the cause. In 93% of lagging chromosomes involving a *cen1*-GFP signal, only a single chromatid was found between the poles, while its sister had already segregated to one pole (Figure 7E). In the remaining 7% of cases where both chromosome I chromatids were found to lag in the middle of the cell, *cen1*-GFP sequences had clearly separated.

Δ*pcs1* mutant cells are hypersensitive to the microtubule-destabilizing agent thiabendazole (data not shown) and are inviable in the absence of Bub1. Furthermore, deletion of *mad2*, needed for the mitotic checkpoint, or *swi6*, which alters the structure of centromeric heterochromatin, causes extremely slow growth in Δ*pcs1* mutants (Supplemental Figure S2E). Pcs1 must therefore be required for an aspect of mitotic kinetochore function that is independent of Bub1, Mad2, or Swi6. The phenotype of Δ*pcs1* mutants could conceivably be caused by a failure to suppress merotelic attachment.

Deletion of *pcs1* did not alter the production of four-spored asci, although it did reduce spore viability from 89.4% to 65.6%. Interestingly, during meiosis I, only very few anaphases with lagging chromosomes were observed in Δ*pcs1* cells (3% compared to 0% in wild-type). Furthermore, heterozygous *cen1*-GFP segregated reductionally in 95% of cells (Supplemental Table S1). However, during meiosis II, 17% of late anaphases contained lagging chromosomes and the rate of meiosis II nondisjunction was greatly elevated. Sister centromeres segregated to the same rather than to opposite spindle poles in 10% of cells, which is never observed in wild-type cells (Supplemental Table S1). These data imply that Pcs1 (unlike its budding yeast homolog Csm1) is not required for segregation of homologs during meiosis I, but is needed for faithful sister chromatid segregation during mitosis and meiosis II.

Discussion

The cosegregation of sister centromeres during meiosis I is fundamental to the production of haploid gametes. The physical basis for this must be found at centromeres/kinetochores (Paliulis and Nicklas, 2000). To understand this process, it is clearly necessary to identify the meiosis-specific proteins involved. Though Rec8 in *S. pombe* is both present at meiosis I centromeres and necessary for preventing sister biorientation, it is also present during meiosis II. Moreover, Rec8 is also essential for sister chromatid cohesion and in its absence the microtubule binding sites on *S. pombe* centromeres may be insufficiently close for sister kinetochores to be mono-oriented. The Mam1 protein in *S. cerevisiae*, on the other hand, is both present at kinetochores only during meiosis I and essential for their switch from biorientation to monoorientation (Toth et al., 2000).

Partly because Mam1's ectopic expression is insufficient to promote monoorientation in vegetative cells and partly because it is too poorly conserved to identify homologs even in evolutionarily not so distant *S. pombe*, we developed a selection scheme for further mutations causing a "*mam1*-like" phenotype. This led to the identification of Csm1 and thereby its partner Lrs4, both of which are also essential for monopolar attachment of sister kinetochores during meiosis I. Unlike Mam1, which is only expressed during meiosis, both Csm1 and Lrs4 are present in vegetative cells, where they form a tight complex that is concentrated within the nucleolus. Csm1/Lrs4 complexes abandon the nucleolus only during a brief period shortly before the first meiotic division and some of them appear together with Mam1 at centromeres. There are therefore two meiosis I-specific activities that promote cosegregation of sister centromeres in budding yeast: the Mam1 protein, and a form of the Csm1/Lrs4 complex that manages to escape from the nucleolus.

Our data are consistent with the notion that the Csm1, Lrs4, and Mam1 proteins associate with meiosis I centromeres as a ternary "monopolin" complex via an interaction with an as yet unidentified kinetochore factor. Because in *S. pombe* the cohesin subunit Rec8 is required for suppressing biorientation of sister kinetochores during meiosis I, it is conceivable that this factor is cohesin. Consistent with this, we found Mam1 association with *CEN3* sequences (measured by ChIP) to be reduced but not abolished in *spo11*Δ *rec8*Δ cells compared to *spo11*Δ cells (data not shown). However, monopolin could equally well bind to centromere-specific kinetochore proteins like the Cbf3, Ndc80, or Dam1 complexes.

At present, we can only speculate as to the function of monopolin at meiosis I kinetochores. During mitosis (and presumably meiosis II), biorientation of sister kinetochores is mediated by the Ipl1/Aurora B kinase, which is thought to destabilize dysfunctional kinetochore-microtubule attachments (Tanaka et al., 2002) through its phosphorylation of Dam1p (Cheeseman et al., 2002). Though monopolin clearly suppresses sister kinetochore biorientation, it cannot act by simply inhibiting Ipl1 because this protein kinase is presumably essential for the analogous process that biorients homologs during meiosis I. Monopolin might nevertheless prevent Ipl1 from promoting biorientation of sister kinetochores while at the same time help it to promote biorientation of homologs. It need not, however, have any direct influence on Ipl1. As suggested by cytological studies on *Drosophila*, it could instead simply promote the fusion of adjacent kinetochores (Goldstein, 1981).

Our discovery of Csm1 has not only revealed a hitherto unsuspected role for nucleolar proteins in meiotic kinetochore behavior but has also opened up the possibility of studying homologous proteins in other fungi. The genomes of *S. pombe*, *C. albicans*, and *N. crassa* encode proteins with significant sequence identity to Csm1 (Figure 7A). Furthermore, the location of Pcs1 (*S. pombe*'s Csm1 homolog) both within the nucleolus and at centromeres implies that all proteins of this class might be descended from a common ancestor that had roles in both of these locations. Remarkably, Pcs1 is

located at centromeres during mitosis and has an important role during mitotic and meiosis II chromosome segregation, but is apparently dispensable for meiosis I.

This raises two questions. First, what proteins besides Rec8 suppress biorientation of sister kinetochores during meiosis I in *S. pombe*? Second, might Pcs1 and Csm1 share some function despite the very different (indeed complementary) phenotypes caused by their mutation? One major difference between *S. pombe* and *S. cerevisiae* centromeres is that the former interacts with two to four microtubules (Ding et al., 1993), whereas the latter with only a single one (Winey et al., 1995). *S. pombe*, like most other fungi, animals, and plants with complex centromeres, must possess a mechanism that prevents attachment of *one* chromatid kinetochore to microtubules with different orientations (known as merotelic attachment). Having only a single microtubule attachment site, *S. cerevisiae* never encounters this problem during mitosis. It nevertheless does arise during meiosis I, where the two microtubule binding sites on each sister chromatid must act as one. We suggest, therefore, that Pcs1- and Csm1-containing complexes may indeed possess the same fundamental activity; namely, an ability to "clamp together" or coordinate adjacent microtubule binding sites. Whereas Csm1 clamps sister sites, Pcs1 clamps adjacent sites along the same chromatid (Figure 7E). Deletion of *CSM1* would lead to loss of coordination of attachment sites on sister kinetochores and thereby induce a default state, in which sister centromeres biorient. Deletion of *pcs1*, on the other hand, would abolish coordination of attachment sites on the same kinetochore and thereby lead to merotelic attachment. Pcs1's dispensability during meiosis I suggests that other proteins encoded by the *S. pombe* genome clamp together sister attachment sites during this stage of the life cycle, which alleviates a requirement for Pcs1. Identification of such factors will be a key challenge for the future.

Equally curious is our discovery that both Csm1 and Pcs1 reside within the nucleolus for most of the yeast life cycle. There is neither a precedent nor any a priori reason for this phenomenon. It cannot, however, be fortuitous, given the conservation of this duality in *S. cerevisiae* and *S. pombe*. Csm1's functions within nucleoli and kinetochores presumably have something in common, though what this is remains mysterious. It is curious that whereas kinetochores lead chromatids toward opposite poles, rDNA appears to be the last segment of the genome to disjoin (Granot and Snyder, 1991). They act as a chromosome's vanguard and rearguard, respectively. Whether Csm1-like proteins have any role in nucleolar segregation deserves further investigation.

What causes release of Csm1 and Lrs4 only during meiosis I clearly needs to be addressed. The process takes place around the same time as the formation of chiasmata during late pachytene and may correspond to the point at which a yeast cell becomes committed to undergoing meiosis as opposed to mitosis. Other events necessary for meiosis are the formation of at least one chiasma per homolog pair, the protection of centromeric Rec8 from forthcoming attack by separase, and steps to avoid a round of DNA replication between the first and second meiotic divisions. Discovering the signal that triggers the release of Csm1 and Lrs4 from the nucleolus may therefore lead us to what may be a master regulator of the meiotic process.

Experimental Procedures

All experimental procedures were described elsewhere, but detailed descriptions are provided in Supplemental Data available at http://www.developmentalcell.com/cgi/content/full/4/4/535/DC1.

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